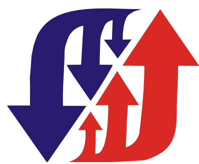

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Hydrothermal Pretreatment of Sugarcane Bagasse: a Tool for Obtaining Hemicellulose Rich Liquors and its Use for Xylanase Production by *Aspergillus Foetidus*

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ABSTRACT

In this work, liquors obtained from Liquid Hot Water (LHW) pretreatment of sugarcane bagasse at different severities were characterized and used as unconventional liquid media for the growth of Aspergillus foetidus *to produce xylanases and arabinofuranosidases. LHW pretreatment liquors were shown to induce high enzyme production, even though high pretreatment severity factor (SF > 3.8) delayed or inviabilized fungal growth. Liquors obtained using low bagasse concentration (1 % m/m) were able to induced high xylanase activity, whereas higher biomass concentrations (9 % m/m) were necessary to induce high arabinofuranosidase production. A number of xylooligosaccharides were identified in the liquors by mass spectrometry, and those may be responsible for xylanase induction. Both solid and liquid fractions obtained after pretreatment induced more xylanase then untreated bagasse, indicating the potential of this technology for improving enzyme production.*

Keywords: Liquid Hot Water pretreatment, hemicellulases, induction, mass spectrometry

INTRODUCTION

Lignocellulose pretreatment technologies for enhancing enzymatic digestibility of cellulose and increasing biofuels production yields have been under intensive study (Silveira et al, 2015). However, less attention is given to biomass pretreatment as a tool for obtaining better substrates for the production of hydrolytic enzymes by lignocellulolytic organisms. Hydrothermal pretreatment of lignocellulose solubilizes a significant portion of the hemicellulose present in biomass, generating a liquid fraction (liquor) rich in soluble hemicellulose fragments and a solid sediment which is less recalcitrant to enzymatic attack and richer in cellulose and lignin (Hu and Ragauskas, 2012). Liquid Hot Water (LHW) pretreatment of sugarcane bagasse (SCB) was studied in this work as a tool for obtaining liquors capable of inducing hemicellulases production (xylanase and arabinofuranosidase) by *Aspergillus foetidus* when used as soluble carbon source.

MATERIALS AND METHODS

Hydrothermal pretreatment was carried as follows: biomass and water at different proportions were added to sealable stainless steel cilinders (300 mL of total internal volume; Swagelok, USA) and incubated in a fluidized sandbath (Tecan SLB-2D; Cole Parmer, USA) at different temperatures and time periods. Cilinders were subsequently cooled in cold water. Pretreated solids were separated from liquor through vacum filtration. Aliquots of pretreatment liquors were stored for compositional analysis. Submerged cultivations of *A. foetidus* were performed in erlenmeyers flasks using liquors supplemented with the following nutrients: KH_2PO_4 (7.0 g/L), K_2HPO_4 (2.0 g/L), $MgSO_4$ (0.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and yeast extract (0.6 g/L) at initial pH 7.0. Aliquots were hervested from cultivations for enzymatic assays. Solids from pretreatment were washed and dried and were also used as substrate for submeged cultivations. Xylanase activity assays were performed by incubating culture supernatant with oat-spelt xylan solution (1 % w/v) at 50 °C for 30 min and the released reducing sugars were measured with dinitrosalicylic acid (DNS) method. Arabinofuranosidase activity was detected using 4nitrophenyl α-L-arabinofuranoside 5 μM as substrate. Reaction was stopped with sodium carbonate 1.0 M and released 4-nitrophenol was detected spectrophotometrically. Reagents were from Sigma (St. Louis, MO, USA).

A Composite Central Rotatory Design (CCRD) 2^3 was used to investigate the effects of incubation temperature (153 – 187 °C), residence time (5 – 55 min) and SCB concentration (1 – 11 % m/m) employed in the pretreatment procedure on the composition of the obtained liquors and the xylanase and arabinofuranosidase enzymatic activities produced by *A. foetidus* when grown on these soluble substrates. One combination of pretreatment conditions was selected, and a comparative study using untreated bagasse, pretreated bagasse, pretreatment liquor and a combination of the former two (whole pretreatment slurry) as carbon sources for enzyme production by *A. foetidus* in submerged cultivations was also carried.

Pretreatment severity factor (SF) was calculated using the following equation: $SF = \log\{t \times \exp[(Ti - Tb)/\omega]\}$, where t is residence time (min), Ti is employed temperature (°C), Tb is base temperature (100 °C) and ω is an adjustment parameter, fixed at 14,75.

Total reducing sugars (TRS), pentoses, glucose and phenolic compounds present in liquors were quantified by DNS method, ferric-orcinol method, glucose oxydase kit and Folin-Ciocalteu reagent, respectively. Liquors were also analyzed by direct infusion mass spectrometry (DIMS), using *eletrospray ionization* (ESI), in both positive and negative modes, and *quadrupole – time of flight* (Q-TOF) analyzer (Maxis 4G, Bruker Daltonics). Principal component analysis (PCA) of the MS spectra was performed. A list of possible liquor components was built and used as base for the identification of ions detected in MS.

RESULTS AND DISCUSSION

The liquors obtained after LHW pretreatment of SCB had their final pH inversely proportional to treatment severity, which can be interpreted as a result of increasing release of organic acids from hemicellulose, mainly acetic acid. Monomeric glucose concentration was very low (0 – 0,039 mg/mL) in all pretreatment liquors. The increase in biomass concentration, pretreatment temperature and residence time from level -1 (3 % m/m, 160 °C and 15 min) to level +1 (9 % m/m, 180 °C, 45 min) had statistical positive effect on the concentrations of TRS, pentoses and phenolic compounds in the resulting liquors, as calculated in factorial design.

Several products of xylan hydrothermolysis were identified by DIMS of liquor samples, such as xylose and a number of xylooligosaccharides (degree of polymerization between 2 to 6), including acetylated ones. The identified xylooligosaccharides may be responsible for the induction of xylanases from *A. foetidus*. Feruloyl-arabinofuranosyl and *p*-coumaroyl-arabinofuranosyl were also detected, indicating that lignincarbohydrate connections are disrupted during LHW pretreatment of SCB. Low molecular weight phenolic compounds derived from guaiacyl (G), syringyl (S) and phydroxyphenyl (H) lignins were also identified, showing that LHW dissolved a portion of lignin present in the SCB. Several ions remain to be identified.

Principal component analysis (PCA) of the MS spectra indicated the clustering of liquors in four main groups: (a) those obtained at high severity treatments (SF > 3.8); (b) at intermediate severities (3,4 < SF > 3,6) with low biomass loading (≤ 3 % m/m); (c) at intermediate severities (3,4 < SF > 3,6) with high biomass loading (≥ 6 % m/m); and (d) at low pretreatment severities (SF < 3). Group (a) was characterized by the presence of xylose (or arabinose), xylobiose, xylotriose, xylopentaose and xylohexaose with different degrees of acetylation, feruloyl-arabinofuranosyl, *p*-coumaroyl-arabinofuranosyl, *p*coumaric acid, benzoic acid and phenylacetaldehyde. Group (b) was characterized by the presence of xylose, hexoses (glucose, mannose, fructose or galctose), feruloylarabinofuranosyl, mannitol (sorbitol) and ion 441.1538 m/z (unidentified), while group (c), by the presence of different phenolic compounds, such as sinapilic acid, guaiacol, hydroxybenzoic acid and siringol. Finally, group (d) was distinguished by the presence of xylitol (or arabitol), mannitol (or sorbitol) and hexose disaccharides (which most likely corresponds to sucrose from sugarcane juice).

Among all combinations of pretreatment variables tested in CCRD, the best pretreatment conditions for xylanase and arabinofuranosidase production by *A. foetidus* grown on resulting liquors were 180 °C for 15 min (corresponding to SF = 3.532) using SCB at 9 % m/m. Under these conditions, xylanase and arabinofuranosidase activities were 3.5 IU/mL (at 6th day of cultivation) and 0.172 IU/mL (7th day), respectively. On the other hand, the use of a much lower SCB concentration (1 % m/m) in a pretreatment of similar severity (170 °C, 30 min, SF = 3.538) was enough to yield high xylanase activity (2.9 IU/mL at 7th day). In spite of xylanase activities, it was observed that high concentrations of SCB (9 % m/m) used in pretreatment were in fact necessary to induce high arabinofuranosidase production. The increase in pretreatment severity and bagasse loading led to increase in enzyme induction up to a certain point, after which the fungus growth and enzyme production were impaired. A long lag phase in *A. foetidus* growth and enzyme production was observed in liquors obtained under severe conditions (SF ≥ 3.8), especially when higher biomass loadings (≥ 6 % m/m) were employed in pretreatment. The high TRS and pentose concentrations in these liquors were not accompanied by increases in enzyme production. Extended lag phases observed in microbial growth on pretreated biomass are usually interpreted as a result of higher concentrations of inhibitory molecules generated during pretreatment, such as phenolic compounds derived from lignin, acetic acid released from hemicellulose and sugar degradation products, including furfural, 5-hydroxymethylfurfural, formic acid and levulinic acid (Jonsson and Martin, 2016). Late enzyme production could also be due to catabolic repression by xylose or other readily metabolizable sugars (Oliveira et al, 2006). Further analysis of liquors by high performance liquid chromatography (HPLC) before and during fungal fermentation will be performed for

the quantification of sugars, acetic acid and sugar degradation products, aiming a better correlation between liquor composition and enzyme production.

When untreated bagasse, pretreated bagasse (washed solids from pretreatment employing 170 °C, 30 min, 1 % m/m), liquor (liquid from the same pretreatment) and whole pretreatment slurry (solids + liquid) were tested as carbon source, *A. foetidus* produced 1.8, 2.5, 2.5 and 3.0 IU/mL of xylanase activity, respectively. In addition to inducing the lowest activity, enzyme production was delayed in untreated SCB, possibly due to the more difficult access of the fungus to its structural carbohydrates. Hydrothermal pretreatment has been shown to increase bagasse specific surface area and, consequently, fungal accessibility (Pereira et al, 2013). The induction of xylanases by pretreated bagasse was attributed to the unreacted xylan fraction remaining in SCB structure and/or to the low-solubility xylooligosaccharides hydrolysed during pretreatment that re-precipitates on the surface of the plant cell wall after pretreatment. Liquors were shown to contain a number of soluble and readily accessible xylooligomers that may take part in the xylanase induction. Whole pretreatment slurry induced the highest xylanase titers, since it contains less recalcitrant structure and soluble inducers.

CONCLUSIONS

This work illustrated that LHW pretreatment of sugarcane bagasse can be used as a tool to increase enzyme production by *A. foetidus*, since both soluble and insoluble fractions obtained after LHW induced more xylanase production then untreated biomass. The use of liquors obtained after pretreatment for the production of industrial enzymes is an alternative way of adding value to the high content of pentoses present in SCB, which cannot be fermented to ethanol by *Saccharomyces cerevisiae*. Besides enzyme induction, liquors may be used as medium for fungus inoculum preparation. It also represents a way of water re-use and partial detoxification of the pretreatment liquid stream for further application in biomass enzymatic hydrolysis within a biorefinerie context. The use of a soluble carbon source is of great interest, since insoluble substrates are less accessible for fungal growth and create operational problems to enzyme production in fermenters.

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